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Role of the CXCR4/CXCL12 Axis in Lymphangioleiomyomatosis and Angiomyolipoma

Debbie Clements,1 Lee J. Markwick,1 Nidhi Puri, and Simon R. Johnson

Lymphangioleiomyomatosis (LAM) is a progressive disease caused by accumulation of metastatic (LAM) cells in the lungs, lymphatics, and the tumor angiomyolipoma (AML). LAM cells have biallelic loss of either tuberous sclerosis complex gene (but predominantly TSC-2) and resultant dysregulation of the mammalian target of rapamycin (mTOR) pathway. Chemokines are associated with neoplastic cell growth, survival, and homing to specific organs and may play similar roles in LAM. Our objective was to study comprehensively the expression and function of chemokine receptors and how their function interacts with dysregulation of the mTOR pathway in LAM and AML. We used RT-PCR and FACS to study receptor expression in primary AML cells and immunohistochemistry to investigate expression in tissues. Chemokine receptor function was analyzed in AML cells by Western blotting of signaling proteins and cell proliferation and apoptosis assays. Primary AML cells, LAM, and AML tissues expressed CCR3, CXCR4, CXC3CR1. In AML cells, their ligands CXCL12, CX3CL1, CCL11, CCL24, and CCL28 caused robust phosphorylation of p42/44 MAPK and Akt. CXCL12 was expressed in type II pneumocytes covering LAM nodules and caused AML cell growth and protection from apoptosis, which was blocked by AMD3100, a CXCR4 inhibitor. The mTOR inhibitor rapamycin, but not AMD3100, inhibited growth of AML tumor xenografts. We conclude that the CXCL12/CXCR4 axis promotes, but is not absolutely required for, AML/LAM cell growth and survival. The Journal of Immunology, 2010, 185: 000–000.
Materials and Methods

**Tissue and cell culture**

Paraffin-embedded sections of LAM lung tissue and AML were collected from biopsy or resection specimens performed for clinical purposes. LAM and AML were confirmed by standard histologic assessment by the reporting pathologist. Primary AML cells were cultured from excess tumor tissue from three patients with TSC-associated AML who were undergoing resection of symptomatic tumor. As previously described, these cells (for phosphorylated S6K1) were isolated from the mTOR pathway (Fig. 1). Consistent with LAM- and AML-derived cells, they have an epithelioid phenotype and strongly express α-smooth muscle actin protein and the mRNA for the melanoma-related protein GP100 (23).

Primary AML cells were cultured and characterized as described (23). Briefly, tumor tissue not required for clinical use was cut into small fragments, treated with type II collagenase, and the resulting suspension seeded into T25 tissue-culture flasks in medium comprising phenol red-free DMEM/F12, ferrous sulfate 1.6 × 10⁻⁸ M, vasopressin 1.2 × 10⁻⁸ U/ml, triiodothyronine 1 × 10⁻⁸ M, insulin 0.025 mg/ml, cholesterol 1 × 10⁻³ M, transferrin 10 pg/ml supplemented with 15% FCS and 5 ng/ml epidermal growth factor [adapted from Goncharova et al. (10)]. Primary AML cells were used at passage 2 to 3. Human airway smooth muscle (HASM) cells were grown from the large airways of surgical resection specimens as described (24). These cells were grown in DMEM with 10% FCS at 37˚C and 5% CO₂ from the large airways of surgical resection specimens as described (24). These cells were grown in DMEM with 10% FCS at 37˚C and 5% CO₂ and were used between passages 4 and 6. SV7tertAML cells were obtained from the American Type Culture Collection (ATCC CRL-2461, Manassas, VA) and cultured in DMEM and 10% FBS. Ethical approvals for the use of LAM and AML tissue, primary AML, and HASM cell culture have been obtained from the Nottingham Local Research Ethics Committee. Informed consent was obtained from all patients.

**Chemokine receptor RT-PCR**

Primers were designed to target all known chemokine receptors (Table I) (25–29). RT-PCR was performed in three primary AML-derived cell lines from two separate donors and the immortalized AML-derived line SV7₄₃AML. Normal HASM cells were also examined as a control. All reactions were repeated from three independent cultures and determined as positive or negative when visualized using ethidium bromide after agarose gel electrophoresis. PCR reactions were run on a Tetratherm 200 (MR Research, Ramsey, MN). The PCR program was as follows: denature for 30 s at 94˚C, anneal for 30 s at 60˚C, and elongate for 30 s at 72˚C for 30 cycles.

**Immunohistochemistry**

All LAM lung tissue sections were from individuals with sporadic LAM. AML tissue sections were from individuals with sporadic LAM or TSC. Primary Ab lists below were used on formalin-fixed paraffin-embedded sections according to the manufacturer’s instructions. Ag retrieval was performed using the microwave/citrate buffer method (30). Detection of the primary Ab was achieved using the Vectastain Mouse/Rabbit/Rat ABC (Vector Laboratories, Peterborough, U.K.), and sections were counterstained with hematoxylin. Abs used were mouse monoclonal anti-CXCR1, CXCR7, CXCR4 (mAbs 171, 172, and 173), CXCR6 and rat monoclonal anti-human CCR3 (R&D Systems, Oxford, U.K.), rabbit polyclonal anti-CX3CR1 (Abcam, Cambridge, U.K.), anti-CXCL12 (R&D Systems), and anti-phospho-p70S6kinase(T389) (Cell Signaling Technology, Danvers, MA). Rabbit and mouse IgG isotype controls were from Sigma-Aldrich (Poole, U.K.), Rat anti-human CMKLR1 Ab and rat IgG2a isotype control were obtained from Dr. Brian Zabel (Stanford University, Palo Alto, CA).

**FACS**

Cells were grown in 75-cm² tissue-culture flasks, removed using enzyme-free cell dissociation media (Sigma-Aldrich), pelleted, resuspended in ice cold 3% BSA/PBS, and fixed in 2% paraformaldehyde. Cells were incubated for 30 min with Abs as described above, washed in BSA/PBS, and incubated with an Alexa Fluor 488-conjugated secondary Ab (Sigma-Aldrich). Flow cytometry was performed using a Coulter Epics Altra HyPerSort System (Beckman Coulter, High Wycombe, U.K.) and data analyzed using WinMDI software (Howard Scripps Institute, http://facs.scripps.edu/software.html). To label intracellular proteins, cells were permeabilized by adding 0.5% saponin to the BSA/PBS.

**CXCL12 ELISA**

Undiluted conditioned media from primary AML cells and SV7₄₃AML cells, grown for 24 h under serum-free conditions, were assayed for secreted CXCL12 by ELISA using the Quantikine Human CXCL12/SDF-1 Immunoassay (R&D Systems) according to the manufacturer’s instructions. The minimum sensitivity of the kit is reported as 47 pg/ml.

**Proliferation assay**

For growth measurement of primary AML cells, we used an MTT reduction assay as described (31). Experimental conditions were run in triplicate wells with at least three independent experiments performed. Data were compared using one-way ANOVA with a Dunnett’s posttest in GraphPad Prism 4 (GraphPad, La Jolla, CA).

**Migration assay**

A scrape wounding assay was used to study cell migration. Primary AML cells were plated at 90% confluency in a 24-well plate and allowed to attach over a 200-μl pipette tip was used to generate two scrape wounds across each well; the cells were then washed with serum-free medium before adding 0.5 ml serum-free medium, medium containing 100 ng/ml recombinant human CXCL12, or medium containing 10% FBS. Wounds were imaged at baseline using a Nikon Diaphot 300 inverted microscope with ×10 objective (Nikon Instruments, Richmond, U.K.), then plates were returned to the culture incubator prior to imaging at 20 h. The number of pixels in the wound area was calculated for defining the outline of the wound in ImageJ http://rsb.info.nih.gov/ij/) (National Institutes of Health, Bethesda, MD), and the difference between the baseline and 20 h was used to determine the rate of wound healing. Eight wounds were examined for each treatment. Postimaging, the 20-h time point cells were incubated with 0.5 mg/ml MTT in serum-free medium for 2 h, and the resulting formazan product solubilized with isopropanol and quantitated at 570 nm on a Thermo Multiskan EX plate reader (Fisher Scientific, Loughborough, U.K.) with Ascent software as an indicator of cell number. Over this time period, neither CXCL12 nor FBS, the positive control, had an effect on cell number, suggesting any changes at this point would be due to migration rather than cell growth.

**Western blotting**

Western blotting was performed as described (23). Abs used were anti-total and phospho-Akt (Pan), p42/44 MAPK, and S6K1 (all from Cell Signaling Technology, Danvers, MA). Sections were incubated with 5% nonfat dry milk in PBS for 2 h, washed with PBS, incubated with primary Ab overnight, washed, and incubated with horseradish peroxidase-conjugated secondary Abs (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary Abs were polyclonal goat anti-mouse Ig HRP (Dako-Cytometry, Cambridge, UK) and goat anti-rabbit IgG (whole molecule) (Sigma-Aldrich). Apoptosis-related proteins were anti-Bcl-2 (Calbiochem, Nottingham U.K.) anti-Mcl1, and anti-Bcl-X₇ (Santa Cruz Biotechnology); loading control was anti-β-actin (Abcam). Filters were digitally scanned and analyzed using Image J version 1.40g (Wayne Rasband, National Institutes of Health). In each experiment, bands were normalized to β-actin. Membranes were stained with Ponceau S for loading. Blots were probed using anti-Bcl-2 and western blots were repeated from three independent cultures and determined as positive or negative. Mean values from three or more independent experiments were compared using one-way ANOVA with a Dunnett’s posttest in GraphPad (GraphPad, San Diego, CA). Recombinant human platelet-derived growth factor-BB, CCL11 (eotaxin), CCL24 (eotaxin-2), CCL26 (eotaxin-3), CCL27 (chemokine T-cell-attracting chemokine), CCL28 (macrophage-attributed epithelial chemokine), CCL5 (RANTES), CXCL16, and CX3CL1 (fractalkine) were obtained from PeproTech (London, U.K.). Recombinant human CXCL12 was obtained from R&D Systems.

**Apoptosis assay**

For the detection of apoptosis, we used an In Situ Cell Death Detection Kit (Roche Diagnostics, West Sussex, U.K.) according to the manufacturer’s instructions. Briefly, cells were plated in eight-well chamber slides and serum starved. After 24 h, cells were treated with 1 μM staurosporine, 200 ng/ml CXCL12, and 100 ng/ml AMD3100 or vehicle for 20 h. In preliminary experiments, these conditions induced apoptosis in ∼50% of AML cells. Cells were then fixed with 4% formaldehyde in PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 10 min, and treated with 50 μl TUNEL reaction mixture in a dark humidified atmosphere for 60 min at 37˚C. Positive controls were treated with 100 ng/ml DNase 1 for 20 min at room temperature prior to the addition of the TUNEL reaction mixture. Negative controls include samples treated with staurosporine but with the addition of label solution without terminal transferase and samples treated with serum-free media instead of staurosporine. Postincubation, cells were stained for 2 min with 1 μg/ml DAPI. The solution was replaced with PBS and cells viewed under an inverted wide-field fluorescence microscope at 200× magnification (Nikon Diaphot 300). Six random fields were counted in each experiment. Images were captured using Metamorph (Mikron Instruments, San Marcos, CA). Each field was counted for FITC and DAPI staining, and the number of apoptotic cells was calculated as a percentage of FITC/DAPI cells. Each experiment was performed in
AML xenograft model

To examine the role of CXCR4 in vivo, we used an AML xenograft model. AML xenografts were established from an immortalized human AML-derived cell line and have been described in detail previously (23). AML xenografts have constitutively active S6K1 and grow in an estrogen-dependent manner. In vivo studies were performed under United Kingdom Coordinating Committee on Cancer Research guidelines. Tumors were excised, weighed, and their clinical condition monitored by a trained observer. Tumor size was measured three times weekly using calipers in two perpendicular dimensions. Mice were terminated individually when the tumor cross-sectional area reached 250 mm² or sooner, which has previously been seen to be below the limit of 10% of initial body weight set by the United Kingdom Coordinating Committee on Cancer Research guidelines. Tumors were excised, weighed, and a portion immediately snap-frozen in liquid nitrogen and stored at −70°C for protein and mRNA extraction; the remainder was preserved for immunohistochemical staining.

Results

Screening chemokine receptor transcripts in AML-derived cells

To study chemokine receptor expression in primary AML cells, we used RT-PCR as our initial screening strategy. The primary AML cells were found to express c kit1 by Western blotting (not shown), but also showed residual phospho-p70S6 kinase after 24 h serum withdrawal, unlike control HASM cells (Fig. 1), consistent with dysregulation of the mTOR pathway. Multiple chemokine receptor transcripts were detected in primary AML cells using primers detailed in Table I (25–29), with CCR1, CCR9, CCR10, CXCR4, and CXCR6 present in >75% of reactions. Normal HASM differed from AML cells in that they did not express CXCR4 (Fig. 1).

Chemokine receptor protein expression

We then examined expression of selected chemokine receptor proteins in primary AML cells. Primary AML cells from three separate donors were examined. Chemokine receptors were chosen for analysis at the protein level if they were expressed in >50% of the initial PCR screen and had potential functions in LAM based on their biology [e.g., chemotaxis: CCR1 and CCR3 (32, 33); homing to lymphatics: CCR7 (34); homing to lungs: CXCR4 (21); proliferation: CXCR6 (35); and CX3CR1 (36)]. FACS demonstrated that CCR3, CXC3CR1, CXCR4, and CXCR6 were all expressed by >20% of AML-derived cells (Fig. 1). Despite the presence of their transcript, CCR1, CCR7, and CXCR7 proteins were not detectable. As we observed that the surface expression of CXCR4 fell rapidly with cell passage (data not shown), cells from P3 or lower were used in these and all other experiments. The same panel of chemokine receptors was then examined by immunohistochemistry in lung tissue of three patients with LAM and in AMLs from three further patients. In keeping with the FACS analysis, CCR3, CX3CR1, CXCR4, and CXCR6 were expressed, whereas CCR1 and CCR7 were absent in all tissues examined (Fig. 2).

Expression of CXCR4 and CXCR6 was predominantly nuclear. To determine that the nuclear localization of CXCR4 was not an artifact of the Ab used, we performed immunohistochemistry using two further Abs raised against different epitopes of the CXCR4 protein. All three Abs gave similar staining patterns in LAM lung and AML sections (not shown). We reasoned that if CXCR4 expression was predominantly nuclear, FACS may underestimate the number of positive cells. We therefore examined CXCR4 expression in permeabilized cells to label intracellular proteins. Permeabilization of the AML cells increased CXCR4 expression from 31.6% to 80.2% of cells, suggesting much of the CXCR4 expression is intracellular. A similar pattern was seen for the related CXCR CXCR6 (Fig. 2).

Chemokine receptor signaling via p42/44 MAPK and Akt in AML-derived cells

To examine the functional activity of these receptors, we examined signaling via p42/44 MAPK and Akt in primary AML cells in response to chemokine receptor stimulation by the recombinant ligands for CCR3 (CCL11, -24, and -28), CX3CR1 (CXC3CL1), CXCR4 (CXCL12), and CXCR6 (CCL16). p42/44 MAPK was chosen due to its reported activity in LAM and cellular proliferation (37). Akt was assayed as it is generally repressed in TSC null cells (38), and we wished to examine if Akt suppression could be overcome by chemokine receptor stimulation. Time courses of activation from 1–120 min were constructed for each chemokine with results from three independent experiments analyzed by densitometry of Western blots. There was robust activation of p42/44 MAPK and Akt by CXCL12, with CXCL12 activation exceeding that achieved by 10% serum (Fig. 3). CX3CL1, CCL11, CCL24, and CCL28 also caused significant activation of Akt and p42/44 MAPK. There was borderline activation of Akt but not p42/44 MAPK by CXCL16 (Table II).

Distribution of CXCR4 and CXCL12 in LAM and AML tissue by immunohistochemistry

Our initial experiments showed that CXCR4 was expressed by most primary AML cells in culture but not normal airway smooth muscle cells. Its ligand, CXCL12/SDF, caused strong activation of p42/44 MAPK and Akt. As CXCR4 has been implicated in tumor growth, we examined CXCR4 and CXCL12 in greater detail. We examined the distribution of CXCR4 and its ligand in paraffin sections of LAM lung nodules and AML. α-Smooth muscle actin was used to identify LAM cells, α-smooth muscle actin–positive LAM cells also stained for phospho[Thr389]–S6K1 consistent with dysregulation of the mTOR pathway (Fig. 4). Tissues from a further seven patients were examined, and all had expression of CXCR4 in the α-smooth muscle actin, phospho-S6K1–positive LAM cells. Expression was both cytoplasmic and nuclear in all cases examined (Fig. 4). CXCL12 was not observed in α-smooth muscle actin, phospho-S6K1–positive LAM cells, but was present in the layer of epithelial cells covering the LAM nodules, previously identified as type II pneumocytes (39) (Fig. 4). Similarly, in sections of AML, LAM cells were α-smooth muscle actin and phospho-S6K1 positive and had predominantly nuclear expression of CXCR4. CXCL12 was absent from LAM cells in AML but present in endothelial and adventitial cells surrounding tumor vessels (Fig. 4).

Relationship between CXCL12 signaling and the mTOR pathway in AML cells

To examine how CXCR4 expression impacts upon the dysregulated mTOR signaling in LAM and AML and the relative contribution of these pathways to LAM cell growth, we treated primary AML cells with CXCL12, the CXCR4 antagonist AMD3100, and the mTOR inhibitor rapamycin. We were unable to detect endogenous expression of CXCL12 by primary AML cells by ELISA of conditioned media. Addition of rCXCL12 resulted in dose-dependent increase in the phosphorylation of p42/44 MAPK and to a lesser extent Akt (Fig. 5).

Consistent with previous observations, AML cells had constitutively phosphorylated S6K1 in the absence of serum. Rapamycin (10 ng/ml) resulted in complete inhibition of S6K1 phosphorylation. Treatment with CXCL12 (200 ng/ml) or AMD3100 (100 ng/ml) alone or in combination had no effect on the activation of S6K1 (Fig. 5).
CXCL12 increases primary AML cell growth in vitro

Activation of p42/44 MAPK is associated with proliferative responses in many cell types; hence, activation of p42/44 MAPK by CXCL12 may contribute to LAM cell growth. As our study has shown that type II pneumocytes adjacent to LAM nodules produce CXCL12, we examined the effect of CXCL12 on the growth of cells derived from AMLs using an MTT assay to examine cell number. Incubation of AML-derived cells with CXCL12 resulted in a small but significant dose-dependent increase in MTT cleavage over a 3-d period (Fig. 5). The chemokines CX3CL1, CCL11, CCL24, and CCL28 had no effect on AML cell proliferation (not shown).

CXCL12 reduces staurosporine-induced apoptosis of AML cells

We next examined if CXCL12 could contribute to the accumulation of LAM cells by exerting a survival influence and protect the cells from apoptosis. CXCL12 (200 ng/ml) treatment of primary AML cells caused a 27% (± 6.4%) reduction in staurosporine-induced apoptosis. Protection from apoptosis by CXCL12 was completely blocked by AMD3100 (100 ng/ml; Fig. 6). As others have described the presence of Bcl-2 and Mcl-1 in LAM cells (40), we next examined if treatment with CXCL12 was associated with an induction of the antiapoptotic proteins Bcl-2, Mcl-1, and the related protein Bcl-XL. Cotreatment of AML cells was associated with a 36% (± 11%) increase in Bcl-2 protein, this did not achieve statistical significance due to the variation between donors. The levels of Mcl-1 and Bcl-XL remained comparatively unaffected (Fig. 6).

CXCL12 does not affect migration of AML cells

Primary AML cells were treated with CXCL12 to determine if a chemotactic effect on these cells could contribute to lung homing of circulating LAM/AML cells. CXCL12 had no significant effect on migration in a wound-repair model (Fig. 7).
Rapamycin, but not AMD3100, reduces growth of AML xenografts

To test if the effect of CXCL12 on growth and apoptosis in AML cells could be a potential therapeutic target for AML or LAM, we studied the effect of AMD3100 on tumor growth in an AML xenograft model (23). As mTOR inhibition has a significant effect on AML-derived cell growth, we studied the effect of rapamycin and AMD3100 alone and in combination in our model. Four groups of six female nude mice received tumor xenografts at day zero and then rapamycin (0.5 mg/kg/d), AMD3100 (1.25 mg/kg/d), rapamycin and AMD3100 in combination, or vehicle. Rapamycin treatment was associated with slower xenograft growth: after 72 d, rapamycin xenografts were 41% of the weight of those from vehicle-treated animals (p, 0.05; Fig. 8). Tumors derived from AMD3100-treated animals were 70% the weight of vehicle treated animals (not significant). AMD3100 also had no added effect on tumor growth in combination with rapamycin (Fig. 8). In a further study, six xenograft-bearing mice per group were treated with either AMD3100 0.25 mg/kg/d or vehicle. The higher dose of AMD3100 again had no effect on xenograft tumor growth (Supplemental Fig. 1).

Table I. PCR primer sequences for chemokine receptors

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FIGURE 2. Expression of chemokine receptors in LAM and AML tissues. A, Immunohistochemical staining for selected chemokine receptor and appropriate control Abs (original magnification X10). B, FACS analysis of permeabilized AML cells for CXCR4 and CXCR6. Figure shows representative histogram of FACS analysis for primary AML cells; black line is IgG control, and shaded area is the chemokine receptor. Figures are mean (± SE) of positive cells from three experiments.
To ensure that loss of CXCR4 expression throughout the study did not account for the absence of effect of AMD3100, xenograft CXCR4 expression was examined by immunohistochemistry. Similar to human LAM and AML tissue, CXCR4 was expressed in tumor xenografts from both groups and was predominantly nuclear. AML tumor xenografts also expressed CXCL12 by immunohistochemistry (Fig. 9). The SV7Tert-immortalized AML cell line used to generate these tumors was also found to secrete CXCL12 in culture (measured at 2564 ± 613 pg/ml by ELISA, not shown).

Discussion
We have demonstrated expression of the chemokine receptors CCR3, CXCR4, CXCR6, and CXC3CR1 in LAM and AML. Furthermore, their ligands caused p42/44 MAPK and Akt phosphorylation in AML cells. CXCL12, the only known ligand for CXCR4, was expressed by type II pneumocytes surrounding LAM nodules in the lung and the vascular endothelial cells of AMLs. In addition to brisk p42/44 MAPK and Akt phosphorylation, treatment of AML cells in vitro with CXCL12 resulted in a modest increase in growth and significant protection from apoptosis. In a xenograft model of AML tumor growth, the CXCR4 inhibitor AMD3100 had no significant affect on tumor growth and did not affect the response to the mTOR inhibitor rapamycin; because both the ligand and the receptor are present on the tumors, the lack of a significant effect of the inhibitor AMD3100 on tumor growth supports the conclusion that the pathway does not have a role in tumor growth. Our findings suggest that it is unlikely CXCL12 is absolutely required for LAM cell growth, and blocking CXCR4 is unlikely to reduce AML or LAM cells growth in lung nodules or lymphatics. However, production of CXCL12 from hyperplastic type II pneumocytes surrounding LAM nodules and perivascular renal cells in vivo could act as a survival or chemoattractant factor for migrating LAM cells favoring their deposition and survival at these sites. Consistent with our findings, another study has reported that CCR3, CXCR4, CXCR6, and CXC3CR1 were present in lung nodules.

Table II. Activation of p42/44 and Akt by chemokines

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<th>Chemokine</th>
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Table shows densitometry results from western blots. All results expressed as ratio over unstimulated.

*p < 0.05; **p < 0.001.
nODULES OF LAM PATIENTS (41). FURTHERMORE, 
50% OF LAM PATIENTS IN THIS STUDY ALSO REACTED WITH ABS AGAINST CCR2, CCR7, CCR10, CXCR1, AND CXCR2 (41). OF THESE, WE DID NOT DETECT THE PRESENCE OF CXCR2 OR CCR2 BY RT-PCR IN OUR CULTURED AML CELLS; DESPITE THE COMMON UNDERLYING MUTATION IN LAM LUNG CELLS AND AML CELLS, SOME DIFFERENCES IN GENE EXPRESSION ARE TO BE EXPECTED. IN ADDITION, ALTHOUGH WE WERE ABLE TO DETECT A TRANSCRIPT IN OUR AML CELLS, CCR1, CCR7, AND CXCR7 PROTEINS WERE NOT DETECTABLE BY IMMUNOHISTOCHEMISTRY IN OUR LAM SAMPLES. THE RT-PCR ASSAY FOR THESE MARKERS IS LIKELY TO BE MORE SENSITIVE THAN IMMUNOHISTOCHEMISTRY, AND WE CANNOT EXCLUDE THE POSSIBILITY THAT EXPRESSION OF CCR1, CCR7, AND CXCR7 PROTEINS WAS BELOW THE DETECTION LEVEL OF OUR IMMUNOHISTOCHEMISTRY ASSAY. IN ADDITION, VARIABILITY IN IMMUNOREACTIVITY TO ANTI-CCR1 AND -CCR7 IN LAM LUNG NODULES HAS BEEN DESCRIBED PREVIOUSLY (41).

FIGURE 5. A, CXCL12 causes a dose-dependent increase in p42/44 MAPK and Akt phosphorylation. B, CXCL12 causes a dose-dependent increase in AML cell growth over 3 d. Graph shows mean (+ SE) of five replicates for three independent experiments. C, AML cell phospho-S6K1 is constitutively active and blocked by rapamycin (10 ng/ml). D, AMD3100 (100 ng/ml) does not affect S6K1 phosphorylation nor does it enhance the inhibitory effect of rapamycin. *p < 0.05 versus control.

LOW, WITH THE ANTIAPOTOTIC PROTEINS BCL-2 AND MCL-1 EXPRESSED BY THE MAJORITY OF LAM CELLS (40). OF POTENTIAL IMPORTANCE IN LAM, BCL-2 WAS COLOCALIZED WITH ESTROGEN RECEPTOR-α AND, IN A FURTHER STUDY USING THE AML XENOGRAPHS MODEL MCL-1, WAS UPREGULATED BY ESTROGEN (23). IN AML CELLS, ALTHOUGH THERE WAS AN INCREASE IN BCL-2 EXPRESSION IN ALL EXPERIMENTS, THIS DID NOT ACHIEVE STATISTICAL SIGNIFICANCE, POSSIBLY AS THE USE OF SMALL NUMBERS OF DONORS AVAILABLE TO PROVIDE PRIMARY CELLS RESULTED IN VARIATION BETWEEN PATIENTS. UNFORTUNATELY, PRIMARY AML CELLS ARE ONLY OBTAINED RARELY AND RAPIDLY LOSE THEIR PHENOTYPE IN CULTURE, MAKING IT DIFFICULT TO STUDY LARGE NUMBERS OF DONORS. ACCEPTING THESE LIMITATIONS, THESE FINDINGS COUPLED WITH OUR CURRENT STUDY MAY SUGGEST THAT CHEMOKINES (AND ESTROGEN) COULD PROMOTE LAM CELL SURVIVAL ...
by the regulation of antiapoptotic proteins possibly via activation of Akt, which, in addition to CXCL12, can be activated by estrogen (23, 43). In TSC-2 null cells, Akt is partially repressed and can be activated by restoration of TSC-2 or rapamycin (44). It is thought that repression of Akt may slow TSC-related tumor growth and have other effects on these cells (38). Restoration of Akt activity by chemokines may therefore be a potential factor in the progression of disease. The failure of CXCR4 antagonism to synergize with mTOR inhibition may reflect both redundancy in stimulatory inputs in LAM but may also represent antagonistic effects on Akt. Our findings suggest that mTOR activation is the dominant growth pathway for LAM and AML. In keeping with this, recent open-label studies have suggested that mTORC1 inhibition with rapamycin can cause regression of TSC-related tumors including subependymal giant cell astrocytomas and AML. However, in these studies in which the drug has been stopped, rapid tumor regrowth occurred (12, 45). Therefore, future targeting of more than one signaling pathway with combination therapies may eventually be required for AML, LAM, and other TSC-related tumors.

Our study raises other possibilities as to the role of CXCR4 in LAM and AML. A number of groups have shown that specific chemokine receptors target metastatic cancer cells to selected organs, and recently it has been reported that CCL2, the ligand for CCR2 and CCR10, induced selective migration of TSC2−/− cells from LAM lung explants (39). CXCR4 could also contribute to LAM cell homing and is associated with lung metastases in breast cancer and malignant melanoma (46) and homing of circulating fibrocytes to the lung in idiopathic pulmonary fibrosis (21). Circulating LAM cells in blood or lymphatics may have high levels of cell-surface CXCR4 with which they target the lungs, kidneys, and lymphatics via CXCL12 gradients. In our study using tumor-derived cells, we were unable to demonstrate a significant effect on migration. In some of these studies, expression of CXCR4 has been associated with strong proliferative (22) and migratory responses to CXCL12 (47, 48), which were not seen in our cells. In these cases, however, CXCR4 was located at the cell surface. In the AML-derived cells in our study, only one third had surface CXCR4 as identified by FACS. It is therefore likely that CXCR4 is performing different functions in the nucleus and at the cell surface. Nuclear CXCR4 in LAM is unlikely to be an artifactual finding, as we verified the nuclear localization with three separate Abs and made consistent observations using the independent technique of FACS. Moreover, nuclear expression of CXCRs is being...
increasingly recognized (49, 50). Although the function of CXCR4 in the nucleus is unknown, it is likely to be biologically important. In breast cancer, nuclear CXCR4 in primary tumors was associated with fewer metastases and improved survival (51). It is also possible that CXCR4 expression may differ in established LAM nodules and AML from circulating LAM cells. The primary AML cells are derived from patients with TSC. Cells were isolated from renal tumor tissue and are likely to result in cultures of neoplastic cells. These cells have a perivascular epithelioid cell phenotype and constitutively active S6K1 despite prolonged serum withdrawal. Although we have not demonstrated that these cells have loss of heterozygosity for TSC-2, we have been careful to confirm our findings in tissues from patients with LAM. As the chemokine receptor profile in our cells and tissues is identical, we feel this approach gives good insight into the human disease.

In summary, we have shown that the chemokine receptors CCR3, CX3CR1, CXC6R, and CXCR4 are expressed in LAM and AML. The CXCR4 ligand CXCL12 is expressed in cells adjacent to LAM and AML cells and in vitro results in p42/44 MAPK. Akt activation, AML cell growth, and reduced apoptosis. It has been recently demonstrated that CXCL12 attenuates apoptosis of human microvascular endothelial cells and rat bone mesenchymal stem cells (52, 53). In addition, it has been shown that CXCR4 signaling inhibits detachment-induced cell death (anoikis) in breast cancer cells, promoting a metastatic phenotype (54). Our data are supportive of this newly emerging antiapoptotic role of the CXCR4/CXCL12 axis and provide an interesting finding on the factors that contribute to the survival and establishment of LAM cells. CXCL12 may act as a survival or chemotactic factor for LAM and AML cells in the lungs and kidneys but is not absolutely required for their growth.

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Disclosures

The authors have no financial conflicts of interest.

References


